

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ :
C12P 21/06, C07H 13/00
A61K 45/05, 39/00, C12N 15/00

A1

(11) International Publication Number: WO 92/03569

(43) International Publication Date: 5 March 1992 (05.03.92)

(21) International Application Number: PCT/US91/05826

(22) International Filing Date: 22 August 1991 (22.08.91)

(30) Priority data:

575,394	29 August 1990 (29.08.90)	US
646,875	28 January 1991 (28.01.91)	US

(71) Applicant: SANGSTAT MEDICAL CORPORATION
[US/US]; 1505-B Adams Drive, Menlo Park, CA 94025
(US).

(72) Inventor: SOULILLOU, Jean-Paul ; 32 ter rue de l'Abbaye,
F-44035 Nantes Cédex 01 (FR).

(74) Agents: BREZNER, David, J. et al.; Flehr, Hohbach, Test,
Albritton & Herbert, Four Embarcadero Center, Suite
3400, San Francisco, CA 94111-4187 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, PL, RO, SE (European patent), SU*.

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROTEIN POLYLIGANDS JOINED TO A STABLE PROTEIN CORE

(57) Abstract

Stable polyligands are provided by preparing fused proteins, where the fused protein comprises a ligand at one terminus and a subunit or a multimeric unit protein at the other terminus, where the fused protein is able to assemble to provide a polyligand. The polyligands find use in modulating physiological processes by inhibiting ligand induced signal transduction by surface membrane protein receptors and/or in the case of μ chain use, by complement mediated killing or any other effector functions. The molecule may be composed solely, of human components to avoid an immune response by the recipient.

BEST AVAILABLE COPY

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU+	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

5 PROTEIN POLYLIGANDS JOINED TO A STABLE PROTEIN CORE

CROSS-REFERENCE TO RELATED APPLICATIONS

10 This application is a continuation-in-part of
application serial number 575,394, filed August 23,
1990.

INTRODUCTIONTechnical Field

15 The field of this invention is proteinaceous
physiologically active polyligands.

Background

20 Many of the activities of mammalian cells are
controlled by the binding of ligands to surface membrane
protein receptors. Thus, DNA replication and cell
proliferation, differentiation, maturation, homing,
metabolism, neuronal signals, and many functional
capabilities can be the result of the binding of one or
more ligands to the surface membrane receptors present
25 on a cell and the transduction of a signal as a result
of this binding. In some situations, such as cancer,
where the cancer cells may proliferate because of
autocrine reactions, there is an interest in inhibiting
the signal transduction. In other situations, such as
30 allograft rejection, it is the initial recognition by
CD4 and CD8 T-cells of the graft as foreign material
which in major part causes the graft to be rejected.
Similarly, allogeneic bone marrow can produce graft vs.
host disease, where allogeneic T-cells are included in
35 the bone marrow implant or autoimmune diseases may
involve T-cells, where inhibition of T-cell
proliferation is desirable.

There are many examples where one wishes to modu-
late cellular response to an available ligand.

Frequently, one wishes to inhibit the ligand-induced signal transduction or render its effect null. Efforts in this direction have employed monoclonal antibodies, there having been numerous reports in the literature in relation to modulating the immune system in animal models. For example, uses of antibodies against the interleukin-2 receptor (IL-2R) have been reported. Monoclonal antibodies targeting peptide receptors have also been used in humans, where a blocking antibody directed against the IL-2R was shown to inhibit the allograft rejection process. Soullillou *et al.*, *Lancet*, (1987), 1:1339-1342; Soullillou *et al.*, *N.E.J.M.*, (1990) 322: 1175-1181. The advantage of this approach is that IL-2R is only expressed on graft recipient lymphocytes activated by donor antigens and not on resting lymphocytes, which are not genetically committed against donor antigens.

For the most part, the reagents used in the treatment of humans have been chimeric or humanized monoclonal antibodies or binding fragments (Fab or (Fab')₂) of monoclonal antibodies directed against membrane receptors. These reagents have several disadvantages *in vivo*, namely relatively low affinity as compared to the ligand itself, usually no or poor effector functions (complement- and antibody-dependent cytotoxicity), or furthermore, to the extent that these proteins are foreign, they elicit the synthesis of host antibodies against isotype or idiotype determinants. "Humanized" antibodies will probably avoid the incidence of antiisotype but not of antiidiotype antibodies, which later behave as blocking antibodies. In addition, several independent monoclonal antibodies are required to give a reasonable chance of reproducing in humans experimental results obtained in an animal model, owing to the possible absence of cross-reactivity.

There is, therefore, a substantial interest in providing alternative bioreagents, which may be used to

inhibit physiological functions or act as mediators of cytotoxicity.

Relevant Literature

- 5 Bacha et al., *J. Exp. Med.*, 167:612-621 (1988) report a reagent prepared by the fusion of IL-2 and diphtheria toxin, while Lorberboum-Galski H. et al., *Proc. Natl. Acad. Sci. USA*, 85:1922-1926, (1988) report the fusion of IL-2 with pseudomonas toxin. These
- 10 reagents have been shown to bind with high affinity to the IL-2R binding site and to have cytotoxic effect. Trauneker et al., *Nature* 339:68-70 (1989); Capon et al., *Nature* 337:525-531 (1989) and Gregerson et al., *Archives of Virology* 111:29-43 (1990) describe CD4-IgH
- 15 or IgL constant region fusion proteins.

SUMMARY OF THE INVENTION

- "Cytomulines" which can be made from compounds which are physiologically naturally occurring in a given
- 20 species, particularly human, are provided, where the cytomulines are characterized by having a plurality of chains naturally linked together, having individual N- and/or C-termini, where each of the chains is extended by fusion to at least a portion of a naturally occurring
- 25 ligand. Particularly, a truncated μ chain of an IgM molecule is fused to at least a binding portion of a ligand, where the ligand provides the N-terminal or C-terminal region. The resulting oligomeric compound mediates physiological effects. Such effects include:
- 30 (1) inhibition of signal transduction in cells carrying the surface membrane receptor for the ligand in question; and
- 35 (2) mediation of complement-dependent cytotoxicity on cells as described in (1). In this case, the complement binding H chain will behave as a "humanized" toxin.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is the DNA sequence (SEQ ID NO. 1) and the encoded amino acid sequence (SEQ ID NO. 2) of the hybrid IL2Mu cDNA. The SalI, BamHI and XbaI sites discussed in the experimental section are indicated;

Fig. 2 is a graph of CTLL2 proliferation as indicated by a number of different fusion proteins; and

Fig. 3 is a graph of C' dependent cytotoxicity for a number of different fusion proteins.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel compositions are provided comprising fusion of chains of naturally occurring polysubunit proteins at their N- or C-termini to the complementary terminus of at least a portion of a ligand which binds to a naturally occurring surface membrane protein receptor. These fusion proteins are referred to as "cytomulines." The resulting product has a plurality of binding sites for binding to the naturally occurring receptor. Furthermore, by employing naturally occurring polysubunit proteins, the modified subunit as a result of the fusion may still be properly processed in an appropriate host organism to assemble the units and provide for the desired polysubunit assembly. Of particular interest and exemplary of polysubunit proteins is the μ chain of IgM.

The subject compositions may be characterized by the following formula:

$(L-SU)_n$

wherein:

L is the ligand or fragment thereof capable of specific binding to the naturally occurring ligand;

SU intends the subunit of a polysubunit protein, where the subunits may be joined together directly or through a central core; and

n is at least 2, preferably at least 4, more preferably at least 6 and may be 10 or more, usually not more than 16, more usually not more than 10.

5 The polysubunit protein will have the following characteristics: (1) it will have at least two chains or subunits, preferably at least about 4 chains, more preferably at least about 6 chains; (2) it will be capable of being bound to a sequence at the N- or C-terminus, without preventing assembly of the
10 polysubunit protein in a host organism. Where desirable, only part of the polysubunit protein may be used. Thus, for IgM μ -chains, for example, the heavy chain domain responsible for interaction with the light chain may be removed; and (3) optionally, it will have
15 effector functions.

These characteristics are demonstrated by the μ chain of IgM or other polyunit naturally occurring proteins, or modified proteins where a cysteine is introduced into the chain of a dimeric or higher order
20 molecule, which cysteines may then be coupled together in vitro to form a higher order oligomer. Exemplary of such proteins are MHC antigens, various members of the immunoglobulin superfamily, β -galactosidase, etc. The μ chain can form an oligomer, usually a decamer, having 10
25 heavy chains. However, by appropriate use of the heavy and light chains with or without the J core, the light, heavy, or both chains may be used for fusion to the ligand binding entity. Processing need not be uniform, so that mixtures of oligomers may be obtained.

30 Such parts of μ heavy chains as provide functions desirable in the novel fused product will be retained. Thus, if domains CH₂ up to CH₄ inclusive are retained (with part or all of domain CH₁ missing) in the fusion product, the latter contains adequate information for
35 assemblage of the novel fused product able to bind complement.

Any of a wide variety of ligands of interest may be employed in the subject invention. Already, there is an

extensive literature of sequences of genes encoding ligands which bind to receptors and are of interest for their physiological activity. These ligands may include such proteins as interleukins 1-7, particularly 1, 2, 3, and 4; cytokines, such as transforming growth factors α and β , tumor necrosis factor, epidermal growth factor, platelet derived growth factor, monocyte-colony stimulating factor, granulocyte-colony stimulating factor, granulocyte, monocyte-colony stimulating factor, erythropoietin, fibroblast growth factor, stem or stem cell growth factor, melanocyte stimulating hormone, (MSH) etc.; interferons- α , β , and γ ; insulin, somatomedin, somatotropin, chorionic gonadotropin, and the like. In addition, major histocompatibility complex antigen binding sequences may serve as ligands.

For the most part, it may be convenient to use the entire ligand. However, in many cases, it may be desirable to use only that portion of the ligand or such extension thereof, which provides for a sufficient level of binding to produce the desired physiological effect. This will vary on a case by case basis, depending on the size of the ligand, whether that portion of the ligand which binds is known, the effect of the fusion of the sequence to the subunit on its activity, and the like. In some instances, one may fuse the two active portions of the molecule by a linking unit, which will usually be fewer than about 25 amino acids. These amino acids may provide a variety of functions, such as allowing for greater hydrophilicity or hydrophobicity at the fusion site, providing for greater access to the binding entity, or the like. Alternatively, one or more mutations, e.g. substitutions, deletions or insertions, may be introduced, usually, not more than 3 mutations. For instance, some modifications in the sequence of the μ chain can modify the complement binding capacity. It is possible that a ligand may have greater affinity when attached to the C- or N- terminus of the truncated

chain. In this situation, the corresponding ligand will be attached to the appropriate terminus.

The subject compositions may be prepared by

5 (1) Construction of a fusion gene coding for the subject composition. For this purpose, employing known genes for the two portions of the subject molecule.

10 (2) Expression of the fusion gene in cells. Thus, in the case of immunoglobulin fusions, genes coding for the immunoglobulin chains (heavy and/or light) may be freed of those sequences coding for the variable region and desirably the first constant region. Usually gene sequences present in the fusion gene will include those coding for the second portion of the constant region. By introducing suitable restriction enzyme sites
15 surrounding the sequences coding for the binding entity and the polysubunit protein, or those parts of these proteins to be retained in the fused product, strategies can be elaborated to link the sequences together in the correct reading frame. The resulting fused sequence
20 will carry an initiation codon, so that the fused gene may be expressed. The two sequences may be brought together by ligation, using PCR, recombination, or the like. Reports describing uses of such techniques abound in the literature. The particular manner in which the
25 two portions of the fused molecule are joined is not critical to this invention and will vary depending upon the particular building blocks employed.

The various manipulations of the genes may be carried out in an appropriate cloning vector, there
30 being a large number of such vectors readily available which provide for high efficiency of cloning, isolation, and replication. These vectors may be illustrated by pBR322, pUC series, and their derivatives. The cloning vectors will be characterized by having an appropriate
35 replication system, a marker for selection, usually one or more antibiotic resistance genes, and one or more polylinkers, which allow for ease of introduction and excision of the sequences being cloned. After each

step, the integrity of the sequence may be checked by restriction enzyme mapping, sequencing, or the like.

DNA sequences coding for dimeric proteins may be modified by in vitro mutagenesis in the region coding for C-proximal amino acids so as to code for a single cysteine not present in the originally encoded protein. Such a cysteine should be unable to form an internal disulfide bridge. For example, selecting an MHC molecule, particularly a Class II molecule the genes encoding α - and β - chains may be mutated to introduce cysteine codons. In the proteins expressed from these mutated genes, the introduced cysteines will not form an intramolecular disulfide bridge within a single chain or between the α - and β - chains of a single MHC molecule. Assembly may be achieved in vitro by activating the thiol groups by appropriate chemical modification.

The activated thiol groups will then react to intermolecular disulfide bridges to form oligomers. Other techniques for controlled linking and formation of oligomers may also be used.

In some instances it may be desired to have different ligands in the same oligomer. Where target cells have combinations of receptors, which combination is different from other cells, the higher avidity of the mixed ligand oligomer will provide for greater selectivity. Examples of such situations include resting cells as compared to stimulated cells, e.g., lymphocytes, endothelial cells etc., progenitor cells and mature or more differentiated cells, normal cells and neoplastic cells, and the like.

Once the fused gene has been prepared, it may be inserted into an appropriate expression vector to generate an expression cassette. In many cases the fused gene itself will carry the signals necessary for the initiation and termination of translation (where this is not the case, these signals will be added to the fusion gene).

Signals for initiation of transcription and RNA processing (capping, splicing, polyadenylation etc.) may be provided by the expression vector.

5 The expression/cassette will include a transcriptional and translational initiation region and a transcriptional and translational termination region. The transcriptional initiation region will comprise an RNA polymerase II promoter, a transcriptional start site, optionally an enhancer, in some instances a sequence
10 which provides for inducible transcription, and such other functional sequences as appropriate. For translation, usually the initiation and termination signals will be carried by the fused gene, and represent those carried by the naturally occurring genes used to
15 make the fused gene. In some cases mutation of these sequences to increase efficiency of translation may be carried out. The transcriptional termination region will provide for a polyadenylation site and termination sequence.

20 The expression cassette can be transformed into an appropriate host cell in a variety of ways. It may be maintained in the host. Alternatively, it may be transformed into the host under conditions whereby the expression cassette will be stably integrated into the
25 genome of the host. In either case, it will normally have a marker for selection of the host containing it. Thus, antibiotic resistance may be employed, such as the neomycin resistance gene, which provides resistance to G418.

30 The expression cassette and the marker may be joined in conjunction with a replication system for extra-chromosomal maintenance in the host. For the most part, mammalian replication systems will be obtained from viruses which infect mammalian cells,
35 such as papilloma virus, adenovirus, simian virus 40, vaccinia virus, or the like. Many vectors are available comprising these replication systems, one or more

markers, and a polylinker, ¹⁰ for insertion of the expression cassette.

Transformation of the host cell may be achieved with any convenient technique, such as electroporation, calcium phosphate precipitated DNA, transfection, use of
5 protoplasts, or the like. Methods of transforming mammalian cell hosts are well known in the literature and need not be exemplified here.

Various mammalian host cells may be employed,
10 which are either normal or neoplastic. The cells may be lymphocytic, particularly B-lymphocytic, or non-lymphocytic, depending upon whether the processing of the μ or other chain with glycosylation is of interest. Non-secreting myeloma cell lines expressing the J chain
15 coding gene may be also used if the μ chain is the polyunit protein core. Coexpression of J chain is not required for complement binding by a μ chain polyunit, but can increase it and facilitate the formation of large multimeric species. After transformation into the
20 appropriate host, the cells will be grown in conventional media where the fused protein comprising the μ chain and the ligand will be expressed and assembled to form a decamer of μ chains, so as to provide for a total of 10 ligands. In this manner, one does not
25 require the light chain. Any host cell which is employed, should not produce either heavy or light chains.

In some instances, a signal sequence may be provided which permits processing of the assembled
30 molecule with secretion. The signal sequence may be natural to the ligand, natural to the polysubunit protein, or foreign to both. In employing a signal sequence, care should be taken that the signal sequence is removed or does not interfere with ligand binding.
35 Signal sequence removal may be intra- or extracellular. Where secretion is obtained, assembly may provide for varied orders of oligomer. The oligomer may be modified in vitro to increase the number of subunits of the

oligomer, e.g. oxidation or thiol activation, where disulfide bridging is involved.

5 The subject methodology may find use with any mammalian host, particularly primates, more particularly humans, and domestic animals, such as murine, bovine, caprine, ovine, canine, feline, equine, lagomorpha, etc.

10 The expressed product may be isolated by lysis of the host cells, isolation from the supernatant, extraction of protein, purification using electrophoresis, affinity chromatography, HPLC, or the like. The purified product may then be formulated in a variety of ways for use as a therapeutic agent. The subject product may be formulated in a variety of physiologically acceptable media, such as deionized water, 15 saline, phosphate-buffered saline, aqueous ethanol, or the like. The concentration of the subject compounds will generally range from about 0.01 to 100 mM, depending upon the dosage level, the efficacy of the product, the nature of administration, the purpose of the 20 administration, and the like. Generally, for similar reasons, the dosages will vary widely, ranging from about 1pg/kg of host to about 1mg/kg of host.

25 The subject compounds may also be used in the study of cells in vitro, phoresis to remove particular cells from a mixture of cells, in stimulating cells to proliferate or to inhibit stimulation and analyze the process involved with the stimulation, and the like. Where mixed ligands are involved there is a high probability that both receptors are bound simultaneously, so that the effect of prolonged simultaneous 30 binding may be investigated.

35 The subject compounds may block the signals generated by a ligand binding to its receptor, by inhibiting the internalization of the complex receptor-ligand and/or kill the target cell by complement mediated cytotoxicity or other effector function, such as antibody dependent cytotoxicity (ADCC) or the like. In this way, a wide variety of events may be modulated,

such as mitosis, differentiation, homing, stimulation, and the like. Thus, one may inhibit an immune response by preventing proliferation of T- and/or B-cells, prevent stimulation of T-cells by binding to MHC antigens, etc.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

10 Example I

A cloned cDNA fragment containing sequences coding for the entire constant region of the human immunoglobulin μ heavy chain is used as a template for polymerase chain reaction (PCR) amplification using the following primers:

SEQ ID NO. 3:

1) 5' - CGGATCCGTGATTGCTGAGCTGCCTCCC -3'

20 SEQ ID NO. 4:

2) 5' - CTCTAGAGGGTCAGTAGCAGGTGCCAG - 3'

This leads to production of a DNA fragment containing sequences coding for the C μ 2 - C μ 4 regions inclusive, lying between a BamHI cleavage site and a XbaI cleavage site. Relevant sequences (only one strand of DNA is shown) are:

SEQ ID NO. 5:

30 5' - C GGATCC GTG ATT GCT GAG CTG CCT CCT
 BamHI V I A E L P P

|-----> IgM

35 --- GCT GGC ACC TGC TAC TGA CCC TCTAGAG - 3'
 A G T C Y * XbaI

This fragment is cleaved with BamHI and XbaI and inserted between the BamHI and XbaI sites of the plasmid PKCR α . The resultant recombinant plasmid (pMu)

is transformed into E.coli strain XL•1 and the cells grown in selective media for selection of transformed hosts which are expanded and grown in 2XTY media until the stationary state. The cells are then harvested and the product isolated and purified by standard cesium chloride density gradient techniques. Total RNA from Jurkat cells are used as a template for reverse transcription using oligo dT as primer. The resulting mixture of cDNAs is used as a template for PCR amplification using the following primers:

SEQ ID NO. 6:

1) 5' - CGTCGACTCCTGCCACAATGTACAGG - 3'

SEQ ID NO. 7:

2) 5' - CGGATCCAGTCAGTGTGAGATGATGC - 3'

This leads to the production of a DNA fragment containing sequences coding for human IL-2 (including the peptide sequence) lying between cleavage sites for Sali (N-terminus) and BamHI (C-terminus). (note that the IL2 stop codon has been replaced by a BamHI cleavage sequence. Relevant sequences are (only one strand shown):

SEQ ID NO. 8:

5' - CGTCGACTCCTCCCACA ATG TAC AGG . . .

Sal I M Y R

. . . AGC ATC ATC TCA ACA CTG ACT GGATCC G - 3'

S I I S T L T BamHI

C-Terminal Amino Acid

This fragment is cut by Sali and BamHI and introduced between the Sali and BamHI sites of pMu. The recombinant plasmid is prepared as above for pMu and called pIL2 Mu. This latter plasmid contains a Sali -

XbaI fragment which codes for an IL-2-(C μ 2->C μ 4) fusion protein; the "linker" region between IL2 and μ is the sequence Gly-ser coded for by the BamHI cleavage site sequence (GGATCC)

5 pIL2-Mu contains the fusion gene in the expression vector pKCR α . The fusion gene is under the transcriptional control of the SV40 early gene promoter and enhancer elements while employing the splice and polyadenylation signals from a rabbit β -globin gene.

10 The subject plasmid is transformed into Sp2/0 cells in accordance with conventional techniques. See Junghans, et al., Cancer Research 50:1495-1502. Since the IL2 gene carries with it the signal sequence, the product is secreted self-assembled into the supernate.
15 In addition, assembled product is retained in the Sp2/0 cells. The cells are harvested, lysed using mild alkali, the protein product isolated free of cellular debris and purified.

Effect on IL2 dependent growth of alloreactive
20 T-cell clones is tested for by the procedure described by Lemauff et al., Human Immunol. 19: 53-58, 1987. Effect on leukemic cell lines and leukocyte growth is shown using the same procedure. The potential capacity of the subject composition to interfere in the immune
25 response of recipients of allografts is tested for as described by Peyronney et al., Transplant. Proc. 20; 300-302, 1988 and Souilliou et al. N.E.J.M. 322: 1175-82, 1990. Since human IL2 is cross-reactive with rat IL2 the activity of the subject composition is
30 demonstrated in rats. Target cells are killed by mediation of complement.

Example II

Materials and Methods

35 Standard molecular biology techniques including the polymerase chain reaction, handling of DNA fragments, transfection of COS-1 cells using a DEAE-dextran protocol, and running of SDS-polyacrylamide gels were

essentially as described in "Molecular Cloning, A Laboratory Manual, second edition", edited by Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

5 To assay supernatants containing IL2Mu or IL2Um for proliferation inducing activity, 3000 cells from the IL2 dependent CTL-L2 cell line were cultured for 18h in the presence of either the appropriate supernatant (various dilutions were tested), medium alone, or known amounts
10 of IL2. Cultures were pulsed with 0.5 μ Ci of tritiated thymidine during the last 6h of incubation, and incorporated thymidine isolated on filters using a cell harvester. Filters were placed in vials together with 0.5ml of scintillation fluid and the radioactivity
15 measured using a beta counter.

Complement dependent cytotoxicity.

CTL-L2 cells were incubated with ice cold COS-1 cell supernatant for 30 min and then washed twice
20 before the additions of rabbit complement (50% in RPMI). After 45 min of incubation of 37°C cell viability was assayed either by counting cells under the microscope in the presence of eosin, or by measuring radioactivity released by cells which had been labeled with $\text{Na}_2^{51}\text{CrO}_4$
25 prior to their exposure to supernatants. Briefly, 3×10^6 cells were incubated with 100 μ Ci of $\text{Na}_2^{51}\text{CrO}_4$ for 2h at 37°C and then washed three times. Spontaneous release of radioactivity (SR) was that released when cells (3000/well) were incubated with medium alone,
30 complement alone, or COS-1 cell supernatant alone. Maximum release (MR) was that obtained in the presence of 1% Triton X100. The radioactivity released when a given supernatant was used together with complement = ER. The specific cytotoxic activity = $100 \times (\text{ER} - \text{SR})$
35 divided by (MR - SR).

Fusion protein analysis. COS-1 cells transfected with appropriate DNAs were cultured for 30h in RPMI 1640

medium devoid of methionine and cysteine (Selectamine, Gibco) but containing 5% dialysed fetal calf serum and 100 μ Ci/ml 35 S-labeled methionine and cysteine (Amersham). Supernatants were applied to a column of Affi-Gel 10 (Bio-Rad) coupled with 3mg of a polyclonal goat anti-human IgM (mu chain specific) antibody (Biosys). The immunoaffinity matrix was then washed extensively with PBS containing 1M NaCl and 0.05% Tween 20, then with PBS diluted 10-fold in distilled water. Elution of bound material was carried out using a glycine-HCl buffer (0.2M, pH 2.5) and eluates monitored by radioactivity and optical density (280nm) measurements. Fractions containing eluted material were immediately neutralized with Na₂HPO₄ (0.5M), pooled and dialysed against phosphate buffer (20mM pH 7.0) before concentration using a Speed Vac. SDS-PAGE was carried out according to Laemmli on 4.5 - 16% polyacrylamide gradient slab gels. Before loading, lyophilized samples were heated for 3min at 95°C in sample buffer containing 5% 2-mercaptoethanol. After electrophoresis, gels were equilibrated with Amplify (Amersham), dried and processed for autoradiography at -70°C. Radiolabeled molecular weight standards were from Pharmacia.

25 RESULTS AND DISCUSSION.

Hybrid cDNA construction.

The general strategy for production of immunoglobulin fusion proteins involves the replacement of the immunoglobulin variable region by the protein of interest. The immunoglobulin mu heavy chain constant region was used. The CH2-CH4 domains contain the sequences necessary to bind complement and mediate ADCC, and also the cysteine residues involved in multimerisation of the Immunoglobulin, normally found as pentamers or hexamers. The IL-2 -(CH2-CH4 domain) fusion protein would form multimers which retained their ability to bind the IL2 receptor with high affinity, fix complement, and mediate ADCC.

As a first step for production of the fusion protein, a hybrid IL2 immunoglobulin mu cDNA was prepared. The cDNA population obtained from reverse transcription of total RNA isolated from transiently stimulated Jurkat cells was used as a template for PCR amplification. One primer used corresponded to a region of the 5' untranslated region of the IL2 mRNA, and was linked to a Sall cleavage sequence. The second primer was complementary to the sequences coding for the carboxy terminal amino acids of IL2, and was linked to a cleavage sequence for BamHI. In this way a Sall-BamHI fragment coding for IL2 was obtained, with the stop codon being replaced by the BamHI site. In another set of experiments a partial cDNA coding for a human immunoglobulin mu heavy chain was used as a template for PCR amplification. One primer used corresponded to sequences coding for the first amino acids of the CH2 domain, and was linked to a BamHI cleavage sequence. The other primer was complementary to sequences coding for the carboxy terminal tail of the immunoglobulin, and was linked to an XbaI cleavage sequence. In this way a BamHI-XbaI fragment coding for the CH2-CH4 domains was obtained. The two PCR products were combined via their BamHI sequences to produce the hybrid cDNA sought after. The sequence is provided in Fig. 1. This cDNA should carry all the information necessary to specify the production of a secreted 483 amino acid fusion protein (IL2-Mu) which can form multimers, bind to the IL2 receptor and activate complement. A variant form of the cDNA was also produced by reversing the orientation of a BstEI fragment (see Fig. 1) contained within the immunoglobulin coding sequences. This cDNA codes for a truncated 221 amino acid fusion protein (IL2Um) which lacks part of the CH2 domain and all of the CH3 and CH4 domains, and thus should be unable to form multimers or bind complement, while retaining the ability to bind to the IL2 receptor.

Expression of fusion protein.

The hybrid cDNAs were introduced into the eucaryotic expression vector pKCR α under control of the SV40 early gene promoter. COS-1 cells were transfected with the resulting plasmids or pKCR α and secreted proteins harvested for analysis. These proteins were subjected to affinity chromatography using an anti-IgM resin. Bound proteins were eluted and analyzed by SDS-PAGE under reducing conditions. This analysis of affinity purified proteins from experiments using the IL2Mu and IL2Um cDNAs permitted detection of 64kDa and 39kDa proteins respectively. Neither protein was detected when the pKCR α vector was used for transfection.

Both IL2Mu and IL2Um stimulate cell growth.

Having demonstrated that the hybrid cDNAs can be used to produce either IL2Mu or IL2Um, we wished to determine whether these proteins could bind to the IL2 receptor. To this end, the fusion proteins were tested for their ability to promote the growth of the IL2 dependent murine T-cell line CTL-L2 and lectin-activated human T lymphocytes. Supernatants from COS-1 cells transfected with IL2Mu or IL2Um expression vectors, unlike those from cells transfected with the "empty" expression vector pKCR α , specifically elicited the proliferation of both murine and human activated T-cells.

IL2Mu but not IL2Um binding leads to complement induced cytotoxicity. (Figs. 2 and 3; two different preparations of each of the fusion proteins are tested.)

In the next study it was determined whether the bound fusion proteins could be used to effect complement induced killing specific to those lymphocytes expressing a high affinity IL2 receptor. To this end, ^{51}Cr labelled CTL-L2 lymphocytes were incubated with the reagents to be tested (supernatants from COS-1 cells

transfected with the IL2Mu and IL2Um expression vectors or the pKCR α vector). Rabbit complement was added 45min. later and the cells incubated for a further hour at 37°C. The amount of ^{51}Cr released was then estimated and the percentage of specific cell lysis calculated. Significant lysis of CTL-L2 cells was observed after incubation with IL2Mu containing supernatants and rabbit complement, while no killing over and above that induced by the complement alone was seen when IL2Um containing supernatants and rabbit complement were used. The phenomenon was dose dependent and specific, as IL2-receptor negative cell lines (such as DA-1a mouse cells) were not killed under the same assay conditions.

Following the above procedures, the sequence encoding the IL2 ligand may be replaced with a sequence encoding any other ligand. In some situations it may be desirable to allow for a mixed composition, where some of the chains comprise a ligand for one receptor, while other chains comprise a ligand for a different receptor. Such mixed compositions may find application where the selected receptors are specific for a particular class of cells, so that the targeted population may be restricted to cells of a particular class.

In addition, the fusion proteins should have the unique advantage of not triggering any immune response from the human recipient, both its components are of human natural origin. Thus, the subject compositions may be repeatedly administered, without being inactivated by the immune system, nor inducing an immune response.

It is evident from the above description that the compounds of the subject invention provide for a unique methodology for inhibiting a wide variety of physiological processes. Thus, the multi-ligand compound can bind to a plurality of surface membrane protein receptors, and may in this manner prevent ligand internalization, hinder signal induction or kill the target cell by complement mediation. In this manner,

many processes may be modulated for prophylactic or therapeutic treatment of mammalian hosts.

By employing the subject compositions, by themselves or in conjunction with other drugs, various
5 conditions, such as graft rejection, autoimmune diseases, graft vs. host disease, and tumors, may be treated.

All publications and patent applications mentioned in this specification are indicative of the level of
10 skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be
15 incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended
20 claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Soullillou, Jean-Paul
- (ii) TITLE OF INVENTION: Protein Polyligands Joined To A Stable Protein Core
- (iii) NUMBER OF SEQUENCES: 11
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Cooley Godward Castro Huddleson & Tatum
 - (B) STREET: 5 Palo Alto Square, Suite 400
 - (C) CITY: Palo Alto
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 94306
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/646,875
 - (B) FILING DATE: 28-JAN-1991
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/575,394
 - (B) FILING DATE: 23-AUG-1990
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Rowland Ph.D., Bertram I.
 - (B) REGISTRATION NUMBER: 20,015
 - (C) REFERENCE/DOCKET NUMBER: ATLA-001/01US
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 415-494-7622
 - (B) TELEFAX: 415-857-0663

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1540 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 17..1528

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGACTCCT GCCACA ATG TAC AGG ATG CAA CTC CTG TCT TGC ATT GCA	49
Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala	
1 5 10	
CTA AGT CTT GCA CTT GTC ACA AAC AGT GCA CCT ACT TCA AGT TCT ACA	97
Leu Ser Leu Ala Leu Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr	
15 20 25	
AAG AAA ACA CAG CTA CAA CTG GAG CAT TTA CTG CTG GAT TTA CAG ATG	145
Lys Lys Thr Gln Leu Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met	
30 35 40	
ATT TTG AAT GGA ATT AAT AAT TAC AAG AAT CCC AAA CTC ACC AGG ATG	193
Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met	
45 50 55	
CTC ACA TTT AAG TTT TAC ATG CCC AAG AAG GCC ACA GAA CTG AAA CAT	241
Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His	
60 65 70 75	
CTT CAG TGT CTA GAA GAA GAA CTC AAA CCT CTG GAG GAA GTG CTA AAT	289
Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn	
80 85 90	
TTA GCT CAA AGC AAA AAC TTT CAC TTA AGA CCC AGG GAC TTA ATC AGC	337
Leu Ala Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser	
95 100 105	
AAT ATC AAC GTA ATA GTT CTG GAA CTA AAG GGA TCT GAA ACA ACA TTC	385
Asn Ile Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe	
110 115 120	
ATG TGT GAA TAT GCT GAT GAG ACA GCA ACC ATT GTA GAA TTT CTG AAC	433
Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn	
125 130 135	
AGA TGG ATT ACC TTT TGT CAA AGC ATC ATC TCA ACA CTG ACT GGA TCC	481
Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr Gly Ser	
140 145 150 155	
GTG ATT GCT GAG CTG CCT CCC AAA GTG AGC GTC TTC GTC CCA CCC CGC	529
Val Ile Ala Glu Leu Pro Pro Lys Val Ser Val Phe Val Pro Pro Arg	
160 165 170	
GAC GGC TTC TTC GGC AAC CCC CGC AAG TCC AAG CTC ATC TGC CAG GCC	577
Asp Gly Phe Phe Gly Asn Pro Arg Lys Ser Lys Leu Ile Cys Gln Ala	
175 180 185	

ACG GGT TTC AGT CCC CGG CAG ATT CAG GTG TCC TGG CTG CGC GAG GGG Thr Gly Phe Ser Pro Arg Gln Ile Gln Val Ser Trp Leu Arg Glu Gly 190 195 200	625
AAG CAG GTG GGG TCT GGC GTC ACC ACG GAC CAG GTG CAG GCT GAG GCC Lys Gln Val Gly Ser Gly Val Thr Thr Asp Gln Val Gln Ala Glu Ala 205 210 215	673
AAA GAG TCT GGG CCC ACG ACC TAC AAG GTG ACC AGC ACA CTG ACC ATC Lys Glu Ser Gly Pro Thr Thr Tyr Lys Val Thr Ser Thr Leu Thr Ile 220 225 230 235	721
AAA GAG AGC GAC TGG CTC AGC CAG AGC ATG TTC ACC TGC CGC GTG GAT Lys Glu Ser Asp Trp Leu Ser Gln Ser Met Phe Thr Cys Arg Val Asp 240 245 250	769
CAC AGG GGC CTG ACC TTC CAG CAG AAT GCG TCC TCC ATG TGT GTC CCC His Arg Gly Leu Thr Phe Gln Gln Asn Ala Ser Ser Met Cys Val Pro 255 260 265	817
GAT CAA GAC ACA GCC ATC CGG GTC TTC GCC ATC CCC CCA TCC TTT GCC Asp Gln Asp Thr Ala Ile Arg Val Phe Ala Ile Pro Pro Ser Phe Ala 270 275 280	865
AGC ATC TTC CTC ACC AAG TCC ACC AAG TTG ACC TGC CTG GTC ACA GAC Ser Ile Phe Leu Thr Lys Ser Thr Lys Leu Thr Cys Leu Val Thr Asp 285 290 295	913
CTG ACC ACC TAT GAC AGC GTG ACC ATC TCC TGG ACC CGC CAG AAT GGC Leu Thr Thr Tyr Asp Ser Val Thr Ile Ser Trp Thr Arg Gln Asn Gly 300 305 310 315	961
GAA GCT GTG AAA ACC CAC ACC AAC ATC TCC GAG AGC CAC CCC AAT GCC Glu Ala Val Lys Thr His Thr Asn Ile Ser Glu Ser His Pro Asn Ala 320 325 330	1009
ACT TTC AGC GCC GTG GGT GAG GCC AGC ATC TGC GAG GAT GAC TGG AAT Thr Phe Ser Ala Val Gly Glu Ala Ser Ile Cys Glu Asp Asp Trp Asn 335 340 345	1057
TCC GGG GAG AGG TTC ACG TGC ACC GTG ACC CAC ACA GAC CTG CCC TCG Ser Gly Glu Arg Phe Thr Cys Thr Val Thr His Thr Asp Leu Pro Ser 350 355 360	1105
CCA CTG AAG CAG ACC ATC TCC CGG CCC AAG GGG GTG GCC CTG CAC AGG Pro Leu Lys Gln Thr Ile Ser Arg Pro Lys Gly Val Ala Leu His Arg 365 370 375	1153
CCC GAT GTC TAC TTG CTG CCA CCA GCC CGG GAG CAG CTG AAC CTG CGG Pro Asp Val Tyr Leu Leu Pro Pro Ala Arg Glu Gln Leu Asn Leu Arg 380 385 390 395	1201
GAG TCG GCC ACC ATC ACG TGC CTG GTG ACG GGC TTC TCT CCC GCG GAC Glu Ser Ala Thr Ile Thr Cys Leu Val Thr Gly Phe Ser Pro Ala Asp 400 405 410	1249

GTC TTC GTG CAG TGG ATG CAG AGG GGG CAG CCC TTG TCC CCG GAG AAG	1297
Val Phe Val Gln Trp Met Gln Arg Gly Gln Pro Leu Ser Pro Glu Lys	
415 420 425	
TAT GTG ACC AGC GCC CCA ATG CCT GAG CCC CAG GCC CCA GGC CGG TAC	1345
Tyr Val Thr Ser Ala Pro Met Pro Glu Pro Gln Ala Pro Gly Arg Tyr	
430 435 440	
TTC GCC CAC AGC ATC CTG ACC GTG TCC GAA GAG GAA TGG AAC ACG GGG	1393
Phe Ala His Ser Ile Leu Thr Val Ser Glu Glu Glu Trp Asn Thr Gly	
445 450 455	
GAG ACC TAC ACC TGC GTG GTG GCC CAT GAG GCC CTG CCC AAC AGG GTC	1441
Glu Thr Tyr Thr Cys Val Val Ala His Glu Ala Leu Pro Asn Arg Val	
460 465 470 475	
ACC GAG AGG ACC GTG GAC AAG TCC ACC GGT AAA CCC ACC CTG TAC AAC	1489
Thr Glu Arg Thr Val Asp Lys Ser Thr Gly Lys Pro Thr Leu Tyr Asn	
480 485 490	
GTG TCC CTG GTC ATG TCC GAC ACA GCT GGC ACC TGC TAC TGACCCTCTA	1538
Val Ser Leu Val Met Ser Asp Thr Ala Gly Thr Cys Tyr	
495 500	
GA	1540

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 504 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu	1	5	10	15
Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu	20	25	30	
Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile	35	40	45	
Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe	50	55	60	
Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu	65	70	75	80
Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys	85	90	95	

Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile
 100 105 110
 Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala
 115 120 125
 Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe
 130 135 140
 Cys Gln Ser Ile Ile Ser Thr Leu Thr Gly Ser Val Ile Ala Glu Leu
 145 150 155 160
 Pro Pro Lys Val Ser Val Phe Val Pro Pro Arg Asp Gly Phe Phe Gly
 165 170 175
 Asn Pro Arg Lys Ser Lys Leu Ile Cys Gln Ala Thr Gly Phe Ser Pro
 180 185 190
 Arg Gln Ile Gln Val Ser Trp Leu Arg Glu Gly Lys Gln Val Gly Ser
 195 200 205
 Gly Val Thr Thr Asp Gln Val Gln Ala Glu Ala Lys Glu Ser Gly Pro
 210 215 220
 Thr Thr Tyr Lys Val Thr Ser Thr Leu Thr Ile Lys Glu Ser Asp Trp
 225 230 235 240
 Leu Ser Gln Ser Met Phe Thr Cys Arg Val Asp His Arg Gly Leu Thr
 245 250 255
 Phe Gln Gln Asn Ala Ser Ser Met Cys Val Pro Asp Gln Asp Thr Ala
 260 265 270
 Ile Arg Val Phe Ala Ile Pro Pro Ser Phe Ala Ser Ile Phe Leu Thr
 275 280 285
 Lys Ser Thr Lys Leu Thr Cys Leu Val Thr Asp Leu Thr Thr Tyr Asp
 290 295 300
 Ser Val Thr Ile Ser Trp Thr Arg Gln Asn Gly Glu Ala Val Lys Thr
 305 310 315 320
 His Thr Asn Ile Ser Glu Ser His Pro Asn Ala Thr Phe Ser Ala Val
 325 330 335
 G Glu Ala Ser Ile Cys Glu Asp Asp Trp Asn Ser Gly Glu Arg Phe
 340 345 350
 Thr Cys Thr Val Thr His Thr Asp Leu Pro Ser Pro Leu Lys Gln Thr
 355 360 365
 Ile Ser Arg Pro Lys Gly Val Ala Leu His Arg Pro Asp Val Tyr Leu
 370 375 380

Leu Pro Pro Ala Arg Glu Gln Leu Asn Leu Arg Glu Ser Ala Thr Ile
 385 390 395 400
 Thr Cys Leu Val Thr Gly Phe Ser Pro Ala Asp Val Phe Val Gln Trp
 405 410 415
 Met Gln Arg Gly Gln Pro Leu Ser Pro Glu Lys Tyr Val Thr Ser Ala
 420 425 430
 Pro Met Pro Glu Pro Gln Ala Pro Gly Arg Tyr Phe Ala His Ser Ile
 435 440 445
 Leu Thr Val Ser Glu Glu Glu Trp Asn Thr Gly Glu Thr Tyr Thr Cys
 450 455 460
 Val Val Ala His Glu Ala Leu Pro Asn Arg Val Thr Glu Arg Thr Val
 465 470 475 480
 Asp Lys Ser Thr Gly Lys Pro Thr Leu Tyr Asn Val Ser Leu Val Met
 485 490 495
 Ser Asp Thr Ala Gly Thr Cys Tyr
 500

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGATCCGTG ATTGCTGAGC TGCCTCCC 28

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTCTAGAGGG TCAGTAGCAG GTGCCAG

27

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Val Ile Ala Glu Leu Pro Pro
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCT GGC ACC TGC TAC TGACCCTCTA GAG
Ala Gly Thr Cys Tyr
1 5

28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Gly Thr Cys Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGTCGACTCC TGCCACAATG TACAGG

26

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGATCCAGT CAGTGTGAG ATGATGC

27

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..21

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGC ATC ATC TCA ACA CTG ACT GGATCCG

Ser Ile Ile Ser Thr Leu Thr

1

5

28

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Ile Ile Ser Thr Leu Thr
1 5

WHAT IS CLAIMED IS:

- 5 1. A composition comprising at least two subunits covalently joined, wherein said subunits comprise at least a portion of a naturally occurring poly(subunit) protein, which portion of said naturally occurring subunit naturally assembles upon expression in a
10 cellular host, fused to at least a portion of a naturally occurring peptide sequence capable of binding to a naturally occurring receptor.
- 15 2. A composition according to Claim 1, wherein said subunit is an immunoglobulin subunit.
3. A composition according to Claim 2, wherein said immunoglobulin subunit is the μ chain.
- 20 4. A composition according to Claim 1, wherein said subunits are joined by disulfide bridges.
5. A composition according to Claim 1, wherein said receptor is a surface membrane protein.
- 25 6. A composition according to claim 1 wherein said subunit is the μ chain and mediates inhibition of the signal transmission after binding to the corresponding receptor by a blockade of receptor
30 internalization.
7. A composition according to Claim 1, wherein said subunit is the μ chain and mediate complement dependent killing or an effector function of the
35 constant portion of an Ig and is cytotoxic to a cell bearing the specific receptor.

8. A composition according to Claim 1, wherein more than one naturally occurring peptide sequence capable of binding to a naturally occurring receptor is present.

5

9. A composition according to Claim 1, wherein said receptor binds to a hormone or cytokine.

10. A DNA sequence encoding a composition according to Claim 1.

10

11. A DNA sequence according to Claim 10 joined to at least one of a stable replication system or a marker for selection of a cellular host.

15

12. A DNA sequence according to Claim 11, wherein said naturally occurring subunit is an immunoglobulin subunit.

13. An expression cassette comprising a DNA sequence according to Claim 10 joined to and under the transcriptional and translational regulation of a transcriptional initiation region and a transcriptional termination region.

20

14. A cellular host comprising a DNA sequence according to Claim 13.

25

15. A method of producing a composition comprising at least two subunits covalently joined, wherein said subunits comprise at least a portion of a naturally occurring poly(subunit) protein, which portion of said naturally occurring subunit naturally assembles upon expression in a cellular host, fused to at least a portion of a peptide sequence capable of binding to a naturally occurring receptor, said method comprising:
growing a cellular host according to Claim 14 in an appropriate nutrient medium, whereby said

30

35

composition is expressed; and isolating said composition.

5 16. A method of inhibiting the modulation of an intracellular signal, where said signal results from the binding of a ligand to a surface membrane protein receptor on a cell, said method comprising:

10 combining a cellular composition comprising cells comprising said surface membrane protein receptor with a composition comprising at least two subunits covalently joined, wherein said subunits comprise at least a portion of a naturally occurring poly(subunit) protein, which portion of said naturally occurring subunit naturally assembles upon expression in a cellular host, 15 fused to at least a portion of a peptide sequence capable of binding to said surface membrane protein receptor.

20 17. A method according to Claim 16, wherein said naturally occurring poly(subunit) protein is an immunoglobulin subunit.

25 18. A method of treating a host to inhibit proliferation of target cell, said method comprising: administering to said host a composition according to Claim 1, wherein the sequences of said naturally occurring subunit and said naturally occurring peptide are substantially homologous to the native sequences of said host, 30 whereby said composition bind to said target cell and inhibits proliferation.

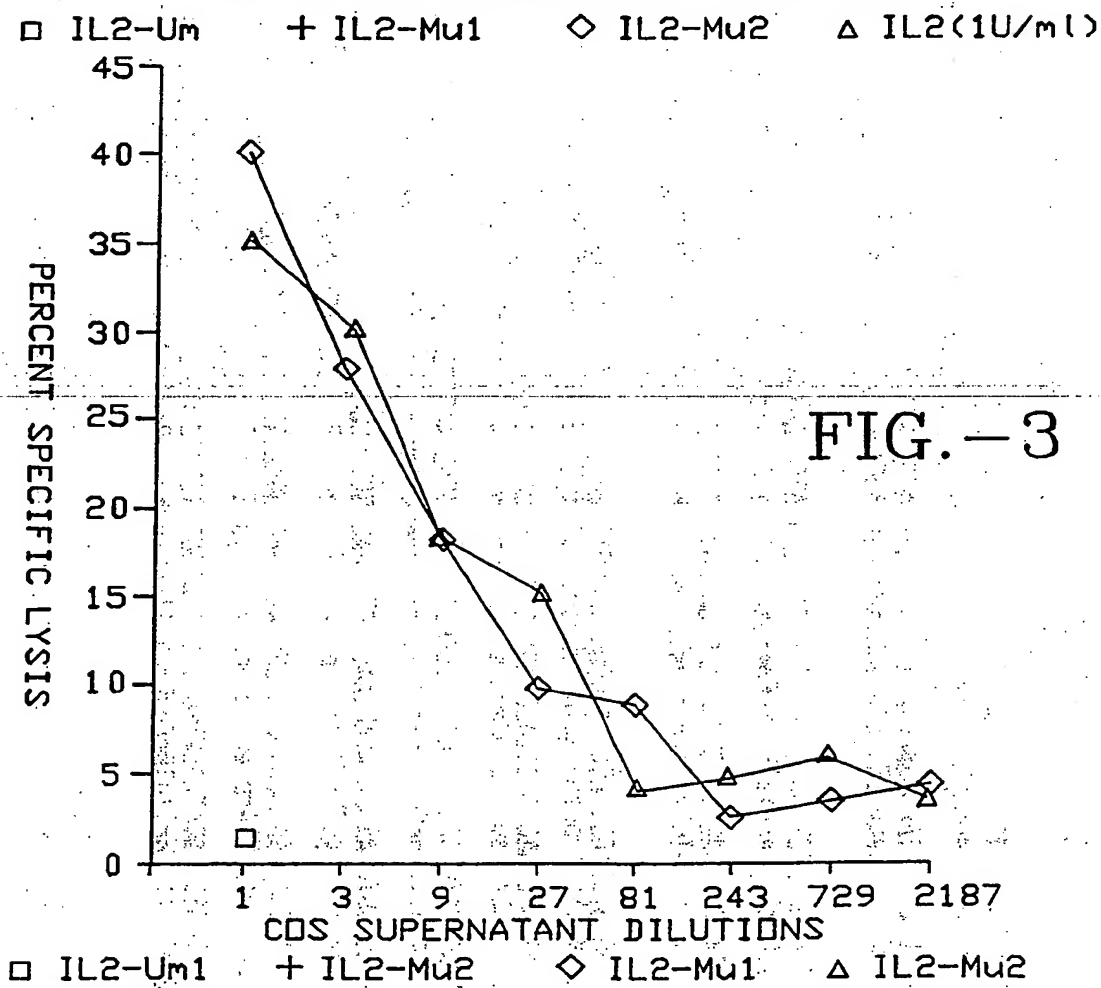
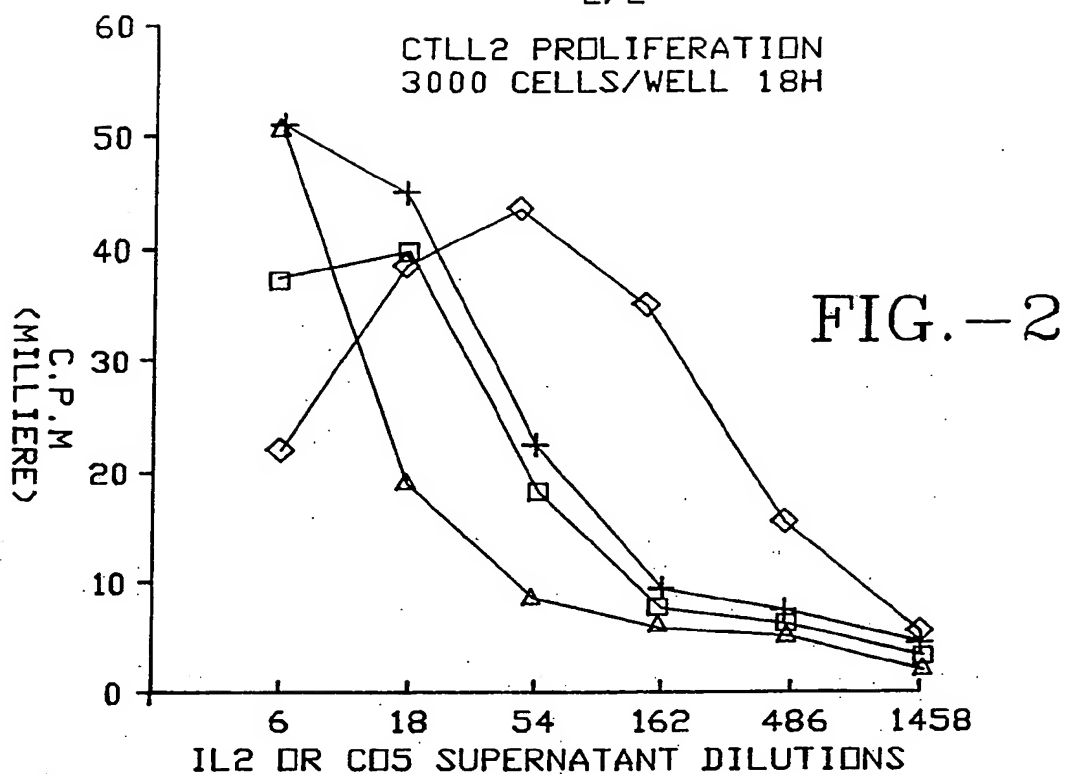
1/2

M

GTCGACTCTGCCACAAATGTACAGGATGCAACTCTCTGCTTGCAATTGCACTAAGTCTTGCACTTGACACAAACAGTGCACCTACTTCAAGTTCTACAAAGAAACACAGCTACAACTGGGA
 MetTyrArgMetGlaLeuLeuSerCysIleAlaLeuSerLeuAlaLeuValThrAsnSerAlaProThrSerSerThrLysLysThrGlnLeuGlnLeuGlnLeuGln
 GCATTACTGCTGGATTACAGATGATTTGAATGGAATTAATACAAAGATCCCAAACTCACCAGGATGCTCACATTAAAGTTTACATGCCAAAGAGGCCACAGAACTGAACACA
 uHisLeuLeuLeuAspLeuGlnMetIleLeuAsnGlyIleAsnAsnTyrLysAsnProLysLeuThrArgMetLeuThrPheLysPheTyrMetProLysLysAlaThrGluLeuLysHis
 TCTTCAGTGTCTAGAAAGAGAACTCAAACTCTGGAGGAAGTGTCTAAATTTAGCTCAAAGCAAAACTTTCACCTTAAGACCCAGGACTTAATCAGCAATAATCAACGTAATAGTTCTGGGA
 sLeuGlnCysLeuGluGluGluLeuLysProLeuGluGluValLeuAsnLeuAlaGlnSerLysAsnPheHisLeuArgProArgAspLeuIleSerAsnIleAsnValIleValIleLeuGln
 ACTAAGGGATCTGAACAACACTCATGTGTGAATATGCTGATGAGACAGCAACCATTTGTAGAAATTTCTGAACAGATGGATTACCTTTTGTCAAAGCATCATCTCAACACTGACTGGATC
 uLeuLysGlySerGlnThrThrPheMetCysGluTyrAlaAspGluThrAlaThrIleValGluPheLeuAsnArgIrpIleThrPheCysGlnSerIleIleSerThrLeuThrGlySer
 CGTGATTGCTGAGCTGCCCTCCCAAGTGAGCGTCTTCGTCACCCCGGAGCGCTTCTCGGCAACCCCGCAACTCCAAAGCTCATCTGCCAGGGCAGGGTTTCAGTCCCCGGCAGAT
 rValIleAlaGluLeuProProLysValSerValPheValProProArgAspGlyPhePheGlyAsnProArgLysSerLysLeuIleCysGlnAlaThrGlyPheSerProArgGlnIle
 TCAAGTGTCTTGGCTGGCGAGGGGAGGAGGAGTGGGGTCTGGGCTCACCACGACCGAGGTGAGGCTGAGGCAAGAGTCTGGGCCACGACCTACAAAGGTGACCAACACTGACCAT
 egInValSerIrpLeuArgGluGluLysGlnValGlySerGlyValThrThrAspGlnValGlnAlaGluAlaLysGluSerGlyProThrThrTyrLysValThrSerThrLeuThrIle
 CAAGAGAGCGACTGGCTCAGCCAFAGCATGTTCACTTGGCGCTGGATCAGAGGGGCTGACCTTCCAGCAGAAATGGGTCTCCATGTGTGCCGATCAAGACACAGCCATCCGGGT
 elysGluSerAspIrpLeuSerGlnSerMetPheThrCysArgValAspHisArgGlyLeuThrPheGlnGlnAsnAlaSerSerMetCysValProAspGlnAspThrAlaIleArgVal
 CTTGGCATCCCCCATCTTTGGCAGCATGTTCTCACCAGTCCACCAAGTTCACCTGGCTGGTGACAGACCTGACACCTATGACAGGTTGACCATCTCTGGACCCGCCAGAAATGG
 IPheAlaIleProProSerPheAlaSerIlePheLeuThrLysSerThrLysLeuThrCysLeuValThrAspLeuThrThrTyrAspSerValThrIleSerTrpThrArgGlnAsnGln
 CGAAGCTGTGAACCCACACCAACATCTCCGAGAGCCACCCCAATGCCACTTCAGCGCGTGGTGAGGCCAGCATCTCGAGGATGACTGGAAATCCGGGGAGAGGTTTCACGTGCAC
 yGluAlaValLysThrHisThrAsnIleSerGluSerHisProAsnAlaThrPheSerAlaValGlyGluAlaSerIleCysGluAspAspTrpAsnSerGlyGluArgPheThrCysTh
 CGTGACCCACAGAGACTGCCCTCGCCACTGAAGCAGACCATCTCCCGGCCCAAGGGGTGGCCCTGCACAGGCCCGATGTCTACTTGTGCCACAGCCCGGGAGCAGCTGAACCTGGC
 rValThrHisThrAspLeuProSerProLeuLysGlnThrIleSerArgProLysGlyValAlaLeuHisArgProAspValTyrLeuLeuProProAlaArgGluGlnLeuAsnLeuAr
 GGAGTCGGCCACCATCAGCTGCTGGTGACGGGTCTCTCCCGGGACGTCTTCGTGCACTGGATGCAGAGGGGGAGCCCTTGTCCCGGGAGAGTATGTACACAGCGGCCCAATGCC
 ggLysSerAlaThrIleThrCysLeuValThrGlyPheSerProAlaAspValPheValGlnTrpMetGlnArgGlyGlnProLeuSerProGluLysTyrValThrSerAlaProMetPr
 BstEI
 TGAGCCCCAGGGCCCGGCTACTTCGCCCACAGCATCTGACCGTGTCCGAGAGGAATGGAAACAGGGGGAGACCTACACCTTCGCTGGTGGCCCATGAGGCCCTGCCCAACAGGGT
 oGluProGlnAlaProGlyArgTyrPheAlaHisSerIleLeuThrValSerGluGluGluTrpAsnThrGlyGluThrTyrThrCysValValAlaHisGluAlaLeuProAsnArgVal
 XbnI
 CACCGACAGGACCGTGGACAGTCCACCGGTAAACCCACCTGTACACCTGTCCTGCTCATGTCCGACACAGCTGGGACCTGTCTACTGACCCCTCTAGA
 IThrGluArgThrValAspLysSerThrGlyLysProThrLeuTyrAsnValSerLeuValMetSerAspThrAlaGlyThrCysTyr

FIG. -1

2/2



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US91/05826

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.: 435/69.1; 536/27; 424/85.8; 935/22, 23

IPC(5): C12P 21/06; C07H 13/00; A61K 45/05, 39/00; C12N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System

Classification Symbols

U.S.

435/69.1; 536/27; 424/85.8; 935/22, 23

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched

APS, Dialog. Bio sci. CAS

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of Document, ¹⁾ with indication, where appropriate, of the relevant passages ²⁾	Relevant to Claim No. ³⁾
Y	US. A, 4,935,352 (Koichi <u>et al</u>) 19 June 1990, See entire document.	1-15
X Y	EP, A, 0,325,262 (Seed) 26 July 1989, 'see entire document.	<u>1-9</u> 10-15, 16-18

* Special categories of cited documents: ⁴⁾

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of the International Search Report

25 November 1991

03 FEB 1992

International Searching Authority

Signature of Authorized Officer

ISA/US

Suzanne Ziska

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.